

## nrDNA ITS 和 cpDNA *rpl16* 基因测序法 对六种鼠尾草的检测<sup>\*</sup>

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**摘要:** 为从鼠尾草属植物中鉴别丹参品种, 采用基因测序方法, 用核糖体核酸内转录间隔区基因 (nrDNA ITS), 编码核蛋白体大亚基多肽 L16 的基因 (*rpl16*) 及叶绿体 DNA 上包含 *tmL* 以及 *trnL* 和 *trnF* 间隔区的区域基因 (*trnL-trnF*) 的序列, 检测六种鼠尾草属新鲜植物。由于 nrDNA ITS 和 *rpl16* 突变率较高, 可以做为 6 种鼠尾草的基源鉴定标记, 依此设计了两对特异引物, 从 6 种鼠尾草中鉴定出丹参 (*Salvia miltiorrhiza*) 和云南鼠尾草 (*S. yunnanensis*)。但 *trnL-trnF* 突变率太低, 未能用于鉴别。商品干燥中药材因加工和储藏的方式致使 DNA 降解严重, 基因测序法难于应用。

**关键词:** 鼠尾草属; ITS 基因; *rpl16* 基因; *trnL-trnF* 基因

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## Identification of *Salvia* Species by nrDNA ITS and cpDNA *rpl16* Sequence Analyses

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**Abstract:** Three DNA regions were sequenced for testing six fresh plant samples of *Salvia* species. These three DNA regions were nrDNA ITS (nuclear ribosomal DNA internal transcribed spacer), chloroplast *rpl16* (the gene encoding ribosomal protein L16), and *trnL-trnF* (the cpDNA region comprising the *trnL* and the intergenic spacer between *tmL* and *trnF*). The results showed that the nrDNA ITS and *rpl16* genes could provide novel information for origin identification of *Salvia* species. Due to their higher mutation rates of these 2 gene markers, *Salvia* species-specific primers were designed and *S. miltiorrhiza* and *S. yunnanensis* were identified. The *trnL-trnF* gene expressed low mutation rate, it could not identify the species. Since the damage of DNA by the pretreatments of the dry roots of Chinese herbs, it is hard to apply the molecular markers to commercial samples for identification.

**Key words:** *Salvia*; ITS; *rpl16*; *trnL-trnF*

The dried root and rhizome of *Salvia miltiorrhiza* Bunge (Danshen) has been used for hundreds of years

as a traditional Chinese medicine (TCM), (Pharmacopoeia Commission of PRC, 2005). Danshen is used

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mostly for its cardioprotective and anti-atherosclerotic effects, but its components have been found to possess diverse potential medicinal activities, including the inhibition of HIV-1 integrase (Abd-Elazem *et al.*, 2002). Although only *S. miltiorrhiza* was recorded in the Chinese Pharmacopoeia, the dried root and rhizome of other *Salvia* species such as *S. yunnanensis* C. H. Wright, *S. przewalskii* Maxim., *S. trijuga* Diels, *S. digitaloides* Diels, *S. flava* Forrest ex Diels and other species have also been used and called Danshen. All these wild *Salvia* are normally not cultivated in large scales by GAP (Good Agriculture Practice) and sometimes mixed-up with *S. miltiorrhiza* due to the similar morphology and similar efficacy. Extracts from different species had different potencies for developing drugs. In the progress of TCM modernization and for further development of this valuable herb medicine, it is of critical importance to accurately record the source of plant materials and standardize the herb species and the medicinal effects, so that the qualities of herb products will be well controlled. Since molecular identification is under investigation and development recently, many molecular markers, especially sequences of fast-evolving DNA regions, have been developed for plant taxonomic studies at the species level (Shaw *et al.*, 2005). We made a preliminary test to evaluate the feasibility of this method, using the nrDNA ITS region, chloroplast *rpl16* gene and *trnL*-*trnF* region of the cpDNA as molecular markers to identify the six *Salvia* species which are used as Danshen in Yunnan province of China.

## 1 Materials and Methods

### 1.1 Plant materials

All fresh *Salvia* samples as listed in Table 1, were collected in the summer of 2005 in Yunnan Province. The fresh samples were dried with silica gel in 12 hours and kept cold for long time

storage. Voucher specimens have been deposited in the Herbarium of the Institute of Botany, Chinese Academy of Sciences, Beijing (PE). Commercial samples of *S. miltiorrhiza* Bunge were bought from Tongrentang drug store of Beijing.

### 1.2 DNA extraction, PCR amplification and sequencing

Total DNA was extracted from silica gel dried leaves using the CTAB method following the protocol of Rogers and Bendich (Rogers and Bendich, 1988) and used as template in polymerase chain reaction (PCR). Similar total DNA extraction method was applied to commercial samples except that PVP (polyvinylpyrrolidone) was added to improve the quality of DNA. The nrDNA ITS region was amplified with primers ITS1N (5'-GTC GTA ACA AGG TTT CCG TAG G) modified from White *et al.* (1990) and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC), the *rpl16* gene was amplified with primers *rpl16F* (5'-CCC/T TTC ATT CTT CCC CTA TGT TG) and *rpl16R* (5'-GCT ATG CTT AGT GTG CGA CTC GTT G) (Small *et al.*, 1998) and the *trnL*-*trnF* region with 5' *trnL*<sup>UAAF</sup> (5'-CGA AAT CGG TAG ACG CTA CG) and *trnF*<sup>GAA</sup> (5'-ATT TGA ACT GGT GAC ACG AG) (Taberlet *et al.*, 1991). The PCR amplification was carried out in a volume of 25  $\mu$ l, containing 5-50 ng of DNA template, 6.25 pmol of each primer, 0.2 mM of each dNTP, 0.75 units of ExTaq DNA polymerase (TaKaRa, Dalian, China). Amplifications were conducted in a Tgradient 96 U thermocycler (Biometre, Göttingen, Germany) with PCR regimes as follows: for ITS, one cycle of 4 min at 70°C, 4 cycles of 40 sec at 94°C, 20 sec at 55°C and 2 min at 72°C, followed by 30 cycles of 20 sec at 94°C, 20 sec at 55°C and 2 min at 72°C with a final step for 10 min at 72°C; for *rpl16* and *trnL*-*trnF*, they were the same except for the annealing temperature at 50°C instead of 55°C. The PCR products were purified using the DNA Fragment Quick Purification/Recover Kit (DingGuo, Beijing). Sequencing reactions were performed with the same primers listed above. After precipitation in 95% EtOH and 3M NaAc (pH 5.2), the sequencing products were separated on a MegaBACE 1000 automatic DNA sequencer (Amersham Biosciences, Buckinghamshire, UK). For nrDNA ITS and *rpl16*, three to six individual plants per species were sequenced and only one individual plant was sequenced for *trnL*-*trnF*, all with both forward and reverse primers.

Table 1 Plant materials and GenBank Accession Nos. of the sequences

Taxa	Voucher	Locality	GenBank Accession Nos.	
			ITS	<i>rpl16</i>
<i>S. yunnanensis</i> C. H. Wright	NO. 201757	Kunming, Yunnan, China	EF014344	EF014350
<i>S. miltiorrhiza</i> Bunge	NO. 2017260	Beijing, China	EF014345	EF014351
<i>S. przewalskii</i> Maxim	NO. 2017266	Lijiang, Yunnan, China	EF014346	EF014352
	NO. 2017272	Deqin, Yunnan, China	EF053400	EF053401
<i>S. trijuga</i> Diels	NO. 2017254	Lijiang, Yunnan, China	EF014347	EF014353
<i>S. digitaloides</i> Diels	NO. 2017269	Lijiang, Yunnan, China	EF014348	EF014354
<i>S. flava</i> Forrest ex Diels	NO. 2017262	Lijiang, Yunnan, China	EF014349	EF014355

### 1.3 Data analysis

Sequence alignments were made with Clustal X 1.81 (Thompson *et al.*, 1997) and refined manually. Genetic distance between *Salvia* species was estimated with Mega 3.1 (Kumar *et al.*, 2004) based respectively on the ITS region and the *rpl16* gene, using Kimura's two-parameter model (Kimura, 1980).

## 2 Results and Discussion

The quality of total DNA of commercial samples was not good enough for amplifying. All PCR products were amplified from total DNA of fresh samples. We obtained about 630 bp for the ITS region, 800 bp for *rpl16* and 775 bp for *trnL*-*trnF*. Sequence alignments of the former two DNA regions are shown in Fig. 1 and Fig. 2, respectively. The ITS region consists of the 5.8S coding region and two internal transcribed spacers (ITS1 and ITS2). GenBank accession numbers assigned for the sequences we determined are listed in Table 1.

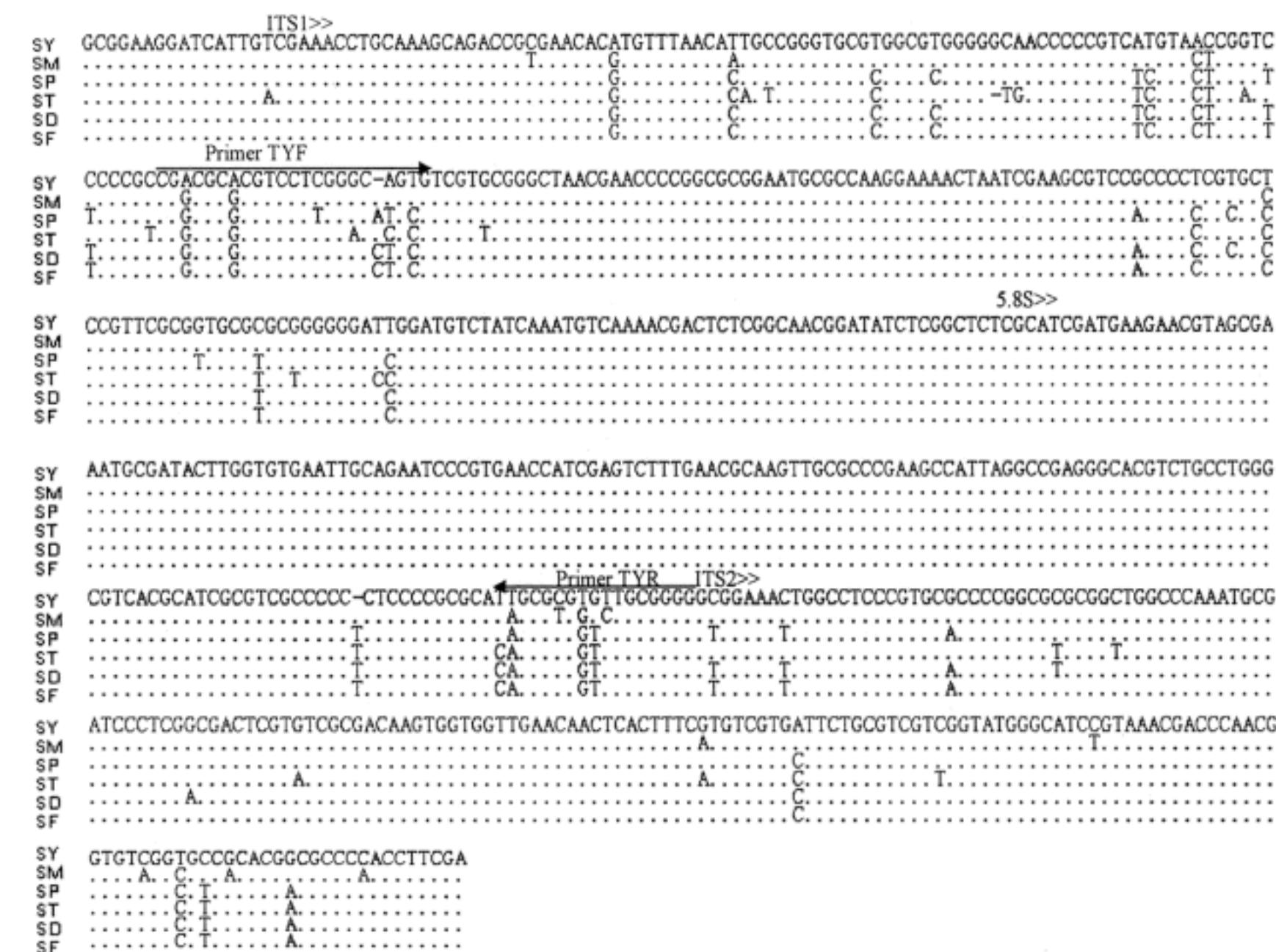


Fig. 1 Sequence comparison of the nrDNA ITS region among the six *Salvia* species. Dot indicates identical nucleotide or gap as in the uppermost sequence. Hyphen indicates an introduced gap. Arrow indicates the position of species-specific primer.

Abbreviations: SD: *S. digitaloides*; SF: *S. flava*; SM: *S. miltiorrhiza*; SP: *S. przewalskii*; ST: *S. trijuga*; SY: *S. yunnanensis*

Among the six *Salvia* species, sequences of the *trnL*-*trnF* region were nearly identical while those of the ITS region and the *rpl16* gene were more variable (Fig. 1, 2). No intraspecific variation was detected, although some species, like *S. przewalskii*, were represented by several individuals collected from different locations. The interspecific sequence divergence was higher in the ITS region (0.005 - 0.065) than in the *rpl16* gene (0.004 - 0.023) (Tables 2, 3), and each *Salvia* species was found to have a unique sequence in the two DNA regions. This indicates that nucleotide sequences of DNA regions like nrDNA ITS and *rpl16* could provide novel information for origin identification of *Salvia* species due to their higher mutation rate. Since the *trnL*-*trnF* region did not show enough variation, it might discount its use as a molecular marker for authentication of *Salvia* species in general.

SY ACTACAGAGCTGGACGTGAGAGTTCTTCATCCAGCTCTCGCGAATAAAAGGATTCCAATTGAATTGAATTAGCTATTAGAACATT  
 SM ..... C .....  
 SP ..... C .....  
 ST ..... C .....  
 SD ..... C .....  
 SF ..... C .....  
 SY TTTATTTAGAAAAAATTTCTTTCTAACGTCTAATAAAATCTTATTGTCTTTATCCGAGTTACCAATTCAAAAAAAA—GAAGGTTTCG  
 SM ..... A ..... A .....  
 SP ..... A ..... A .....  
 ST ..... A ..... T .....  
 SD ..... A ..... T .....  
 SF ..... A ..... T ..... Primer RYF-1 →  
 SY CGGGCGAATATTTACTCTTGCATCTCTATTCAGTCCTAGCACTTATTCGTCTTTCAAACAAAATAGATGAATTATTTCGGTTATTCGCCAT  
 SM ..... G ..... C ..... — ..... C ..... C .....  
 SP ..... G ..... C ..... — ..... C ..... C .....  
 ST ..... G ..... C ..... — ..... C ..... C .....  
 SD ..... G ..... C ..... — ..... C ..... C .....  
 SF ..... G ..... C ..... — ..... C ..... C .....  
 SY CCTAAGTAGTGAATCATTAAAGATTCTGTTATTAATATTTAAATAAAATCTTTGCATTACAGGTTCCCTCGTTTACCACTTCGTACTAGATGATT  
 SM ..... ATA ..... C .....  
 SP ..... C .....  
 ST ..... C .....  
 SD ..... C .....  
 SF ..... C .....  
 SY GGTAGAATTCTACAATGGAGCTCATATCCAAATTATTCTGAGTCACCTCTTAATCTTATTGACTCAAAGCTCTGAGTTTTCTTCTTCTA  
 SM ..... T ..... T ..... G .....  
 SP ..... T ..... T ..... G .....  
 ST ..... T ..... T ..... G .....  
 SD ..... T ..... T ..... G .....  
 SF ..... T ..... T ..... G .....  
 SY TAAGAAATTCAATTCTTCAATTGTATGAATCAATTAGTATTGATGCTTTTACACTGTCTTTATGAGATGACTCATAGACCTCCATTGGAATTGT  
 SM ..... C ..... A .....  
 SP ..... A ..... A .....  
 ST ..... A ..... A .....  
 SD ..... A ..... A .....  
 SF ..... A ..... A .....  
 SY ATATCATTGATATTCTTCTCTTCTCATCCTCCATTATCCACACCTTATCTTCCGTAAATTCTTAAAAAGTTAACGTTAATTATT  
 SM ..... A ..... G ..... A .....  
 SP ..... A ..... T ..... G ..... A ..... G .....  
 ST ..... A ..... T ..... G ..... A ..... G .....  
 SD ..... A ..... T ..... G ..... A ..... G .....  
 SF ..... G ..... T ..... G ..... A ..... T ..... G .....  
 ← Primer RYR  
 SY CAGTTGCTACAAAGATATGACTGATTTAACATATTGACTGGTTCTTAGATCTAGATAATATGAAGT  
 SM .....  
 SP .....  
 ST .....  
 SD .....  
 SF .....

Fig . 2 Sequence comparison of the *rpl16* gene among the six *Salvia* species . Dot indicates identical nucleotide or gap as in the uppermost sequence . Hyphen indicates an introduced gap . Arrow indicates the position of species-specific primer .

Abbreviations: SD: *S. digitaloides*; SF: *S. flava*; SM: *S. multiorrhiza*; SP: *S. przewalskii*; ST: *S. trijuga*; SY: *S. yunnanensis*

Table 2 Genetic distance between six *Salvia* species according to the ITS region sequences

	1	2	3	4	5	6
1. <i>S. yunnanensis</i>						
2. <i>S. miltiorrhiza</i>	0.029					
3. <i>S. przewalskii</i>	0.053	0.051				
4. <i>S. trijuga</i>	0.065	0.060	0.046			
5. <i>S. digitaloides</i>	0.055	0.053	0.008	0.041		
6. <i>S. flava</i>	0.050	0.048	0.006	0.040	0.005	

To discriminate *Salvia miltiorrhiza* from *S. yunnanensis* and other related species in Yunnan fastly and efficiently, species-specific primers were further designed based on sequences of the ITS region and the *rpl16* gene . We found that the primer pair TYF (5'-CGA CGC ACG TCC TCG GGC AGT) and TYR (5'-

Table 3 Genetic distance between six *Salvia* species according to the *rp16* gene sequences

	1	2	3	4	5	6
1. <i>S. yunnanensis</i>						
2. <i>S. miltiorrhiza</i>	0.005					
3. <i>S. przewalskii</i>	0.017	0.015				
4. <i>S. trijuga</i>	0.023	0.020	0.019			
5. <i>S. digitaloides</i>	0.015	0.015	0.008	0.019		
6. <i>S. flava</i>	0.016	0.013	0.007	0.017	0.004	

GGG CGC AAC ACG CGC AAT) (Fig . 1) specifically amplified the ITS region of *S. yunnanensis* at the annealing temperature of 55 (Fig . 3: A), while primers RYF-1 (5'-TCA AAC AAA ATA GAT GAA T) and RYR (5'-AGT TAA ATC AGT CAT ATC) (Fig . 2) specifically amplified the *ml16* gene of *S. miltiorrhiza*

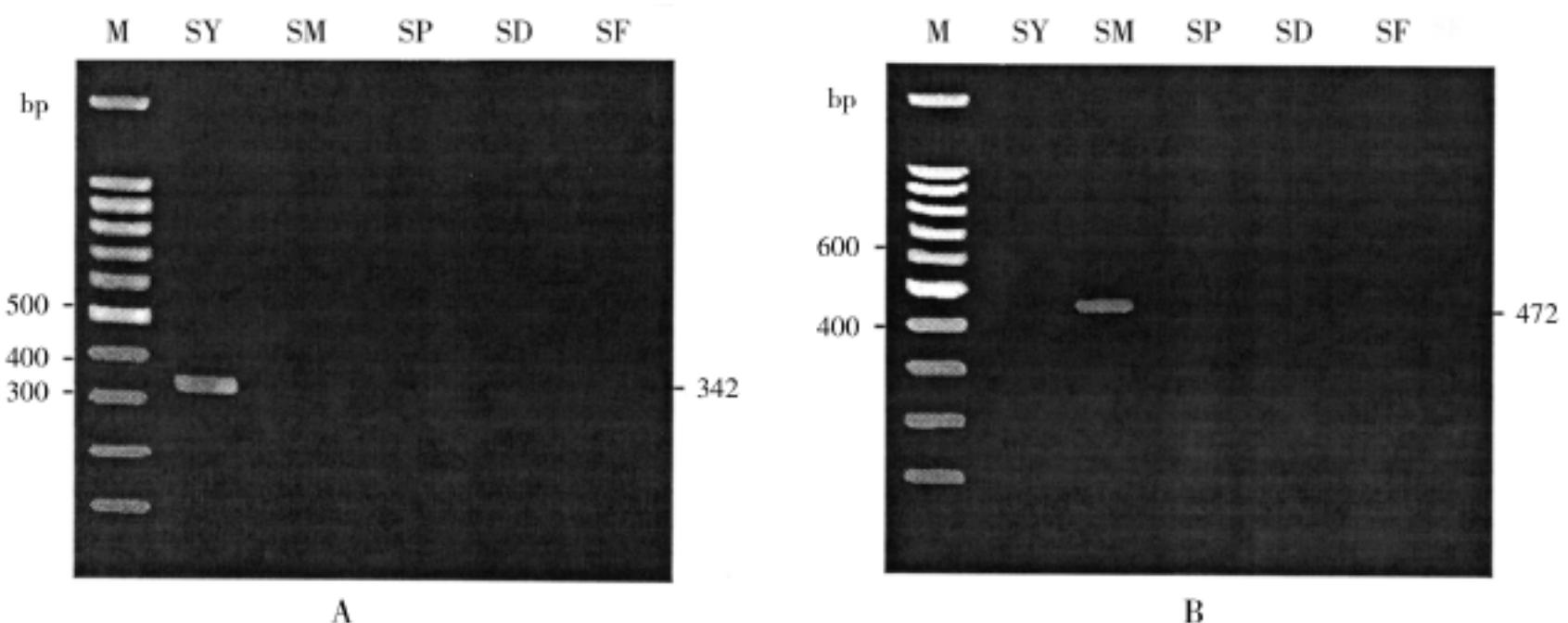


Fig. 3 Specific amplification of parts of the nrDNA ITS region (A) and the *rpl16* gene (B). Separation of the fragments was achieved by electrophoresis on 1.5 % agarose gels, and bands were visualized with GoldViewTM (SBS Genetech, Beijing). M: 1.5 kb DNA marker.

Abbreviations: SD: *S. digitaloides*; SF: *S. flava*; SM: *S. miltiorrhiza*; SP: *S. przewalskii*; ST: *S. trijuga*; SY: *S. yunnanensis*

at the annealing temperature of 40 (Fig. 3: B). Thus, primers TYF and TYR differentiated *S. yunnanensis* from the other five *Salvia* species and primers RYF-1 and RYR offered an alternative method for the Chinese Pharmacopoeia to authenticate *S. miltiorrhiza*.

One of the problems that restrain the development of traditional Chinese medicine is the authentication of raw material in the field and on the commercial markets. Ways based on DNA sequencing may offer an alternative and more reliable method for the identification of Chinese medicinal materials (Zhao *et al.*, 2002; Cao and Komatsu, 2003; Chen *et al.*, 2002; Wang *et al.*, 2005; Lau *et al.*, 2001; Sun *et al.*, 2004; Ding *et al.*, 2002; Kojoma *et al.*, 2002; Hosokawa *et al.*, 2004; Xu *et al.*, 2006) and this will lead to another set of standardization of traditional Chinese medicine. However, DNA-based polymorphism may not handle the identification all by itself. Morphological and chemical studies are indispensable since most Chinese medicinal materials on the market have been processed. The condition of storage and processing of raw materials often result in DNA degradation. All the samples tested were live plants collected in the field and dried by silica gel soon after. Samples bought from the market had also been tested in our laboratory, but unfortunately, the quality of DNA was not qualified to be amplified. With the fact of DNA degradation, species-

specific primers aimed for shorter DNA fragments should be designed, which may give satisfactory results.

In conclusion, we have been able to molecularly distinguish some *Salvia* species used as TCM plants with two markers. However, since it's hard to apply the markers to the commercial samples, maybe molecular identification based on sequence analyses couldn't shoulder the task of identifying Chinese medicinal materials all alone. DNA-based polymorphism may offer an efficient choice for authenticating medicinal herbal species for researchers, but further investigation is still in need for developing its market value.

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#### References:

- Abd-Elazem IS, Chen HS, Bates RB *et al.*, 2002. Isolation of two highly potent and non-toxic inhibitors of human immunodeficiency virus type 1 (HIV-1) integrase from *Salvia miltiorrhiza* [J]. *Antiviral Res.*, **55** (1): 91—106
- Cao H, Komatsu K, 2003. Molecular identification of six medicinal *Curcuma* plants produced in Sichuan: evidence from plastid *trnK* gene sequences [J]. *Acta Pharm Sin.*, **38** (11): 871—875
- Chen YQ, Wang N, Zhou H *et al.*, 2002. Differentiation of medicinal *Cordyceps* species by rDNA ITS sequence analysis [J]. *Planta*

Med, **68** (6): 635—639

Ding XY, Xu LS, Wang ZT *et al.*, 2002 . Authentication of stems of *Dendrobium officinale* by rDNA ITS region sequences [J] . *Planta Med*, **68** (2): 191—192

Hosokawa K, Shibata T, Nakamura I *et al.*, 2004 . Discrimination among species of *Papaver* based on the plastid *rpl16* gene and the *rpl16-rpl14* spacer sequence [J] . *Forensic Sci Int*, **139** (2 - 3): 195—199

Kimura M, 1980 . A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences [J] . *J Mol Evol*, **16** (2): 111—120

Kojoma M, Kurihara k, Yamada K *et al.*, 2002 . Genetic identification of Cinnamon (*Cinnamomum* spp.) based on the *trnL- tmF* chloroplast DNA [J] . *Planta Med*, **68** (1): 94—96

Kumar S, Tamura K, Nei M, 2004 . MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment [J] . *Brief Bioinform*, **5** (2): 150—163

Lau DT, Shaw PC, Wang J *et al.*, 2001 . Authentication of medicinal *Dendrobium* species by the internal transcribed spacer of ribosomal DNA [J] . *Planta Med*, **67** (5): 456—460

Pharmacopoeia Commission of PRC, 2005 . Pharmacopoeia of the People's Republic of China, part [R] . Beijing: Chemical Industry Press, 52—53

Rogers SO, Bendich AJ, 1988 . Extraction of DNA from plant tissues [A] . In: Gelvin SB, Schilperoort RA, eds . Plant Molecular Manual [M] . Dordrecht, Netherlands: Kluwer Academic Pub ., **A6**: 1—10

Shaw J, Edgar BL, John TB *et al.*, 2005 . The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis [J] . *Am J Bot*, **92** (1): 142—166

Small RL, Ryburn JA, Cronn RC *et al.*, 1998 . The tortoise and the hare: choosing between noncoding plastome and nuclear *Adh* sequences for phylogenetic reconstruction in a recently diverged plant group [J] . *Am J Bot*, **85** (9): 1301—1315

Sun Y, Fung KP, Leung PC *et al.*, 2004 . Characterization of medicinal *Epimedium* species by 5S rRNA gene spacer sequencing [J] . *Planta Med*, **70** (3): 287—288

Taberlet P, Gielly L, Pautou G *et al.*, 1991 . Universal primers for amplification of three non-coding regions of chloroplast DNA [J] . *Plant Mol Biol*, **17** (5): 1105—1109

Thompson JD, Gibson TJ, Plewniak F *et al.*, 1997 . The CLUSTAL-X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools [J] . *Nucleic Acids Res*, **25** (24): 4876—4882

Wang CZ, Li P, Ding JL *et al.*, 2005 . Identification of *Fritillaria pallidiflora* using diagnostic PCR and PCR-RFLP based on nuclear ribosomal DNA internal transcribed spacer sequences [J] . *Planta Med*, **71** (4): 384—386

White TJ, Bruns T, Lee S *et al.*, 1990 . Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics [A] . In: Innis M, Gelfand D, Sninsky J *et al.* PCR Protocols: A Guide to Methods and Applications [M] . San Diego: Academic Press, 315—322

Xu H, Wang ZT, Ding XY *et al.*, 2006 . Differentiation of *Dendrobium* species used as " Huangcao Shihu " by rDNA ITS sequence analysis [J] . *Planta Med*, **72** (1): 89—92

Zhao ZL, Wang ZT, Xu LS *et al.*, 2002 . Studies on the molecular markers of rhizomes of some *Alpinia* species [J] . *Planta Med*, **68** (6): 574—576